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Biochimica et Biophysica Acta 1553 (2002) 158–170

BIOCHIMICA ET BIOPHYSICA ACTA

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Review

Quinol:fumarate oxidoreductases and succinate:quinone oxidoreductases: phylogenetic relationships, metal centres and membrane attachment

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Received 20 June 2001; received in revised form 12 September 2001; accepted 12 October 2001

Abstract

A comprehensive phylogenetic analysis of the core subunits of succinate:quinone oxidoreductases and quinol:fumarate oxidoreductases is performed, showing that the classification of the enzymes as type A to E based on the type of the membrane anchor fully correlates with the specific characteristics of the two core subunits. A special emphasis is given to the type E enzymes, which have an atypical association to the membrane, possibly involving anchor subunits with amphipathic helices. Furthermore, the redox properties of the SQR/QFR proteins are also reviewed, stressing out the recent observation of redox-Bohr effect upon haem reduction, observed for the *Desulfovibrio gigas* and *Rhodothermus marinus* enzymes, which indicates a direct protonation event at the haems or at a nearby residue. Finally, the possible contribution of these enzymes to the formation/dissipation of a transmembrane proton gradient is discussed, considering recent experimental and structural data.

Keywords: Succinate:quinone oxidoreductase; Quinol:fumarate oxidoreductase; Redox-Bohr; Phylogeny; Amphipathic helix

1. Introduction

The succinate:quinone oxidoreductases (SQRs) and quinol:fumarate oxidoreductases (QFRs) are composed of a soluble domain attached to the membrane through one or two polypeptides. The soluble domain is located on the internal membrane side, and is built by two subunits: subunit A, containing a covalently linked FAD, and subunit B, with three iron-sulphur centres, a $[2Fe\text{-}2S]^{2+/1+}$ (centre 1), a $[3Fe\text{-}4S]^{1+/0}$ (centre 3) and a $[4Fe\text{-}4S]^{2+/1+}$ (centre 2) (a detailed description of these enzymes may be found in the editorial overview at the beginning of this Special Issue). The structural arrangement of these domains is such that it assures the efficient electron transfer between the catalytic domain, the flavoprotein, through the iron-sulphur subunit, and the membrane domain, where the electron donor/acceptor (the quinol or quinone) is located [1,2].

In this review, we will analyse in a comprehensive way the subfamilies of succinate:quinone oxidoreductase and quinol:fumarate oxidoreductase, on the basis of (i) an extensive comparison of the amino

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0005-2728/02/$ – see front matter
PII: S0005-2728(01)00239-0

acid sequences, (ii) specific features of the metal centres (type and ligands), and (iii) distinct membrane anchors. The novel subfamily of these enzymes (type E), so far exclusively isolated from archaea, which were not detailed in previous excellent reviews on this subject (e.g. [3,4]) is extensively described. Recent data on the redox properties of the haem centres will also be discussed in the framework of the proposed mechanisms for succinate oxidation/fumarate reduction coupled to the dissipation/formation of the proton gradient.

2. Phylogenetic analysis

The two core subunits, forming the hydrophilic domains, are quite conserved in Bacteria, Archaea and eucaryotic mitochondria. This conservation, as previously observed, includes not only the domains harbouring the redox and catalytic centres, but also the overall extension of the proteins. The amino acid sequences of SQR and QFR subunits A and B retrieved from public primary sequence databases were aligned (not shown) and the corresponding phylogenetic trees constructed (Fig. 1). Interestingly, the trees independently obtained for each subunit are in overall almost identical to each other, clearly indicating a common evolution of both subunits (see below).

Inspection of the trees shows that distinct clusters of sequences can be grouped (Fig. 1; these clusters are highlighted by shaded boxes) according to specific features: (i) the type of enzyme; (ii) the type of FeS clusters, including the ligands to the [2Fe-2S]$^{2+/1+}$ centre; (iii) the number and type of anchoring subunits; (iv) the number of haems; and (v) for most cases, identical subunit genomic organisation. Interestingly, the observed clusters are not directly related to the organismal phylogeny, or to the putative function, i.e. as succinate:quinone oxidoreductase or quinol:fumarate oxidoreductases. Table 1 contains a summary of the different characteristics of SQR/QFR according to the clusters obtained in the phylogenetic tree.

The membrane attachment domains were previously classified as types A–D [4]. Recently the A–D type classification was extended to the whole enzyme and the novel subfamily of SQR/QFR (type E) was included [5]. The phylogenetic analysis here performed corroborates this new classification.

Therefore, two large groups can be readily identified. The largest comprises six clusters, and includes the enzymes of types A, B, C and D (Fig. 1). The second group corresponds to the enzymes that lack a canonical membrane anchoring and seem instead to contain anchor subunits with amphipathic helices that assure the interaction with the membrane, classified as type E [5].

In evolutionary terms, and considering the above data, it seems clear that the iron-sulphur and flavin subunits evolved together and have a monophyletic origin. The fact that the phylogenetic trees resulting from the analysis of subunit A and B sequences practically overlap each other indicates that, as far as we can track, throughout evolution both were submitted to identical pressures. In agreement, functional independent proteins with extensive similarities towards subunits A or B were not yet found. As previously suggested, the likely precursors of subunits A and B could have been, respectively, a flavoprotein and a ferredoxin [6]. Additionally, the thiol:fumarate oxidoreductases have a type E anchoring domain (see below) as a C-terminal extension at subunit B [7].

The information necessary to reconstruct the evolution from these precursors cannot be found within the available protein sequences. Thus the functional association between these early precursors of subunits A and B seems to have also been present before the Archaea–Bacteria split, as SQR/QFR enzymes are found in the three domains of life; however, it should be stressed that this could have been achieved by lateral gene transfer. Indeed, the distribution of the archaeal enzymes among the several groups strongly suggests that within archaea, the enzymes do not descend from a common archaeal ancestor, and that several archaea acquired SQR/QFR through lateral gene transfer from bacteria (as recently suggested for the terminal oxygen reductases of the haem-copper superfamily [8]).

With respect to the anchor subunits, the low amino acid sequence identity observed between them, in contrast to the flavo and iron sulphur ones, impairs an adequate estimation of phylogenetic relationships between these subunits. A similar observation is made for the various types of transmembrane *b*-type cytochromes, and may be related to the fact

that these subunits are submitted to a less intense evolutionary pressure. In fact, the maintenance of the secondary structure elements required for transmembrane attachment may be achieved by a quite diverse combination of amino acids. The same may eventually apply to quinone interacting regions, assuming that these essentially require an adequate hydrophobic environment. Mutations at the haem binding regions would be expected to be less well tolerated but also occurred throughout evolution, as shown by the combinations of haems found: none, one or two modifications, which nevertheless may have had considerable functional implications (see Section 4.2).

3. Distribution of the five types of enzymes through the phylogenetic tree

Subunit A is the most conserved subunit. The sequence alignment of all available SQR/QFR showed that the amino acid residues forming the dicarboxylate binding site (see [1]) are strictly conserved in all proteins with the exception of *Campylobacter* (*C.*) *jejuni* SQR, which has a methionine replacing Phe^{141} (*Wolinella succinogenes* numbering). The His, which is covalently bound to FAD, is also conserved in all sequences except for *C. jejuni* that contains an alanine in an equivalent position. The amino acid residues forming hydrogen bonds with the flavin (see [1,2]) are also conserved, although some conservative substitutions are found that are in general preserved within each cluster.

In the iron-sulphur subunit, the conservation is higher in the regions around the iron-sulphur binding sites. The enzymes of types A–D have one iron-sulphur centre of each type (bi-, tri- and tetranuclear), but the third ligand to the binuclear $[2Fe\text{-}2S]^{2+/1+}$ centre is variable, being found a cysteine (the most common ligand), an aspartate or, in one case, a serine. Specific features on these regions are discussed below.

3.1. Type A

Type A enzymes can be subdivided into two differ-

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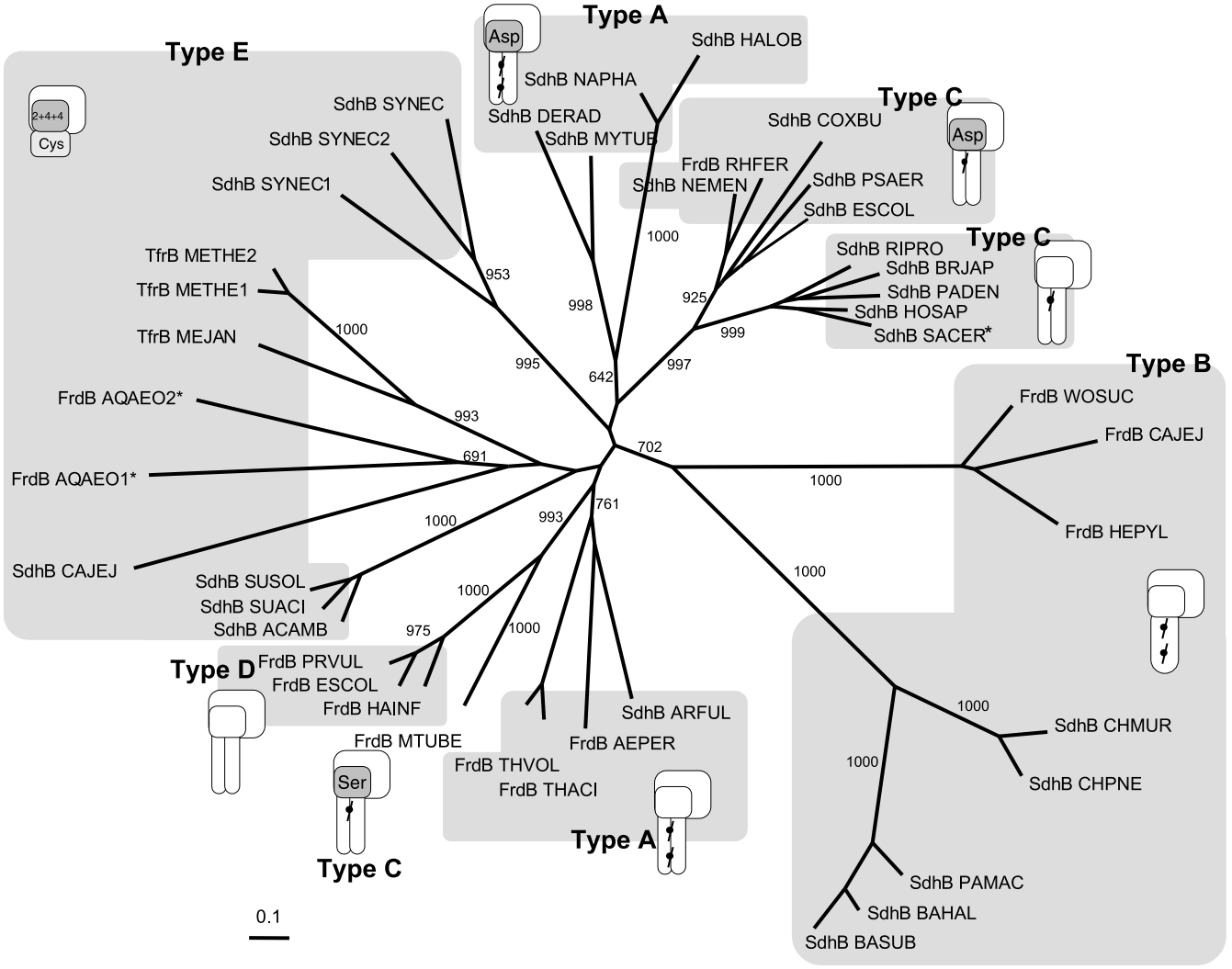
Fig. 1. Core subunit based phylogeny of QFR and SQR. The tree shown was derived from analysis of the FeS subunit sequences. However, a tree with an almost identical topology was obtained using the flavoprotein subunit sequences. Protein sequences were retrieved from public databases using the NCBI protein query tools, and the TIGR genome search utilities. Amino acid sequence alignments were performed with ClustalW using default parameters and manually inspected and adjusted when necessary, using Genedoc. To simplify the analysis and avoid bias, on clusters of sequences highly identical to each other, only a representative number of sequences on each cluster were kept. After this step, a total of 42 distinct sequences were further analysed. The phylogenetic content of the aligned sequences was evaluated using likelihood-mapping analysis [47]. The calculations made using Tree-puzzle [47] showed that tree topology would have a node support of over 80%. Distance-based phylogeny calculations were made on ClustalW, excluding positions with gaps, and using the Kimura distance correction factor implemented in the program. Bootstrap analysis showed that tree clades receive high support values and thus the coherence of the groupings is in agreement with the definition of types A–E of SQR/QFR. The FeS subunits shaded in grey have 'differences' in the FeS clusters: 'Asp' and 'Ser' denote the aspartate and serine, respectively, substituting for the third cysteine in the binuclear cluster, and '2+4+4' denotes the unusual composition of FeS centres in this group, that have a binuclear and two tetranuclear centres. The 'Cys' subunit is the cysteine rich SdhE. ACAMB, *A. ambivalens* (3378541); AEPER, *Aeropyrum pernix* (strain K1) (7521083); AQAEO1, *Aq. aeolicus* (7431704); AQAEO2, *Aq. aeolicus* (7431703); ARFULG, *Ar. fulgidus* (11498290); BAHAL, *B. halodurans* (10175713); BASUB, *B. subtilis* (1770053); BRJAP, *Bradyrhizobium japonicum* (31669725); CAJEJ, *C. jejuni* QFR (6967884) and SQR (6967912); CHMUR, *Chlamydia muridarum* (719098); CHPNE, *Chlamydophila pneumoniae* AR39 (7189996); COXBU, *Coxiella burnetii* (1706242); DERAD, *D. radiodurans* (strain R1) (7473937); ESCOL, *E. coli* K12 SQR (1786943) and QFR (1790596); HAINF, *H. influenzae* (1074114); HALOB, *Halobacterium* sp. NRC-1 (10580826); HEPYL, *H. pylori* (strain J99) (7431698); HOSAP, *Homo sapiens* (9257242); METHER1, *Me. thermoautotrophicum* strain Delta H (2894538); METHER2, *Me. thermoautotrophicum* strain Delta H (7427738), MEJAN, *Methanococcus jannaschii* (1498856); MYTUB, *M. tuberculosis* SQR (2894229) and QFR (1403489); NAPHA, *N. pharaonis* (1524301); NEMEN, *Neisseria meningitidis* MC58 (7226190); PADEN, *Paracoccus denitrificans* (975319); PAMAC, *Paenibacillus macerans*, (1619247); PRVUL, *Proteus vulgaris* (66071); RHFER, *Rhodoferax fermentans* (3273346); RIPRO, *Rickettsia prowazekii* (7431695); SACER, *Saccharomyces cerevisiae* (6322987); SUACI, *S. acidocaldarius* (1654088); SUSOL, *S. solfataricus* (13815660); SYNEC1, *Synechocystis* sp. (1673321); SYNEC2, *Synechocystis* sp. (1652853); SYNEC3, *Synechococcus* PCC7002 (3184554); THACI, *T. acidophilum* (10640317); THVUL, *Thermoplasma volcanium* (13541577); WOSUC, *W. succinogenes* (320458). Proteins are identified by the gi number assigned by NCBI. The designation as QFR and SQR follows that presented in the databases.

Table 1
Classification of the types of SQR/QFR

Type	Iron-sulphur centres					Anchor			Haem		Genomic organisation
	Centre 1[a]			Centre 3		CD	C	EF	b_P	b_D	
	Cys	Asp	Ser	3Fe	4Fe						
A	+			+		+			+	+	*ABCD*
		+		+		+			+	+	*CDAB*, *CDBA*
B	+			+			+		+	+	*CAB*
C	+			+		+			+		*CDAB*
		+		+		+			+		*CDAB*
			+	+		+			+		*ABCD*
D	+			+		+					*ABCD*
E	+				+			+			*ABEF*

The genomic organisation for each type is also presented.
[a]This column reports the third of four ligands of the binuclear centre. In most enzymes the third ligand is a cysteine, but in some cases it can be substituted by an aspartate and there is an example where a serine is the substitute. See also Fig. 1.



ent subgroups, based on the ligand to the [2Fe-2S] centre (Fig. 1 and Table 1): a group in which an aspartate is substituting the third cysteine as a ligand to one of the irons of the binuclear centre (Fig. 2), and a second subgroup containing only archaeal enzymes, in which the binuclear centre is bound by four cysteines, as in most enzymes (Fig. 2).

3.2. Type B

All enzymes from this group form a consistent cluster in the phylogenetic tree (Fig. 1 and Table 1). The proteolytic residue of the transmembrane domain (Glu66 in *W. succinogenes*) that was proposed to be involved in proton uptake during the oxidation of quinol [9] is not conserved in all members of this cluster: while it is conserved in *W. succinogenes*, *C. jejuni* and *Helicobacter* (*H.*) *pylori* [9], in the other type B enzymes, the glutamate is conserved in *Bacillus* (*B.*) *halodurans*, but is replaced by an aspartate (*Bacillus subtilis* [9]), or by other hydrophilic, but not proteolytic, residues.

The SQR and the QFR recently isolated from the bacteria *Rhodothermus marinus* and *Desulfovibrio gigas*, respectively, belong most probably to this type of enzymes, since they were isolated with three subunits, and contain two B-type haems [10,11].

3.3. Type C

As for the type A enzymes, the type C ones can also be subdivided into two subgroups, according to the ligands of the [2Fe-2S] centre: one group of enzymes have an aspartate substituting for the third cysteine, while the other has the four cysteine ligands. There is also one single example, the QFR from *Mycobacterium tuberculosis*, which has a serine substituting for a cysteine as the third ligand to the binuclear centre (Fig. 2). In the subunit A based tree this enzyme occupies a similar position (data not shown).

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FrdB WOSUC  48DPDLNFDFVCRAGICGSCGMMINGRPSLACRTLTKDFE85
SdhB ACAMB  48DPTLAYRASCHMAVCGSCGMKINGEPRLACKTLALDMV85
FrdB MTUBE  47DGTLSFRWSCRMGICGSSGMTINGDPKLACATFLADYL84
SdhB ESCOL  46DPSLSFRRSCREGVCGSDGLNMNGKNGLACITPISALN83

FrdB WOSUC 138EPEVAQEVFELDRCIECGCCIAACGTKIMRED-FVGAA174
SdhB ACAMB 141KPEDQRELWKFEQCIWCGLCVSACPSVKNDPE-FLGPA177
FrdB MTUBE 136TPAELDAFKQFSMCINCMLCYSACPVYALDPD-FLGPA172
SdhB ESCOL 136MPEQREKLDGLYECILCACCSTSCPSFWWNPDKFIGPA173

FrdB WOSUC 196YELIGDDDGVFGCMTLLACHDVCPKNLPLQSKIAYLR-232
SdhB ACAMB 198LKILID--SAWRCTYCYQCFNVCPRDIEPVTTIKKTRA233
FrdB MTUBE 193RDVLAAADGAWACTLVGECSTACPKGVDPAGAIQRYKL230
SdhB ESCOL 194LDGLSDAFSVFRCHSIMNCVSVCPKGLNPTRAIGHIK-230
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Fig. 2. Multiple sequence alignment of the four different types of SdhB/FrdB subunits. Four representative organisms were chosen: WOSUC, *W. succinogenes*; ACAMB, *A. ambivalens*; MTUBE, *M. tuberculosis*; ESCOL, *E. coli* K12 (see legend to Fig. 1).

3.4. Type D

This small group, of which only three examples are so far known, contains two transmembrane subunits, but does not have any haem.

3.5. Type E

The second large group contains all enzymes having one extra cysteine in the binding motif of the trinuclear centre (e.g. Cys^{212} from *Acidianus* (*A.*) *ambivalens* in Fig. 2). This group includes the thiol:fumarate oxidoreductases from methanogens. Accordingly, these enzymes do not have a $[3Fe\text{-}4S]^{1+/0}$ centre, as deduced by EPR studies on intact membranes from the archaeon *A. ambivalens* [12]. This observation was further corroborated by EPR studies on the purified enzymes from *Sulfolobus* (*S.*) *acidocaldarius* [13], *A. ambivalens* [14] and *Sulfolobus tokodaii* (formerly *Sulfolobus* sp. strain 7) [15], and by the determination of the gene sequences of these enzymes. In these cases, the absence of the characteristic EPR resonances for the oxidised $[3Fe\text{-}4S]^{1+/0}$ centre was clear. For the *S. acidocaldarius* SQR evidence was presented for a second $[4Fe\text{-}4S]^{2+/1+}$ centre, but similar EPR resonances could not be observed for the *A. ambivalens* enzyme [14]. Quite interestingly, *A. ambivalens*, when grown anaerobically, appears to express another type of SQR/QFR, containing a canonical $[3Fe\text{-}4S]^{1+/0}$ centre, as deduced by the EPR spectra of intact membranes from cells grown under these conditions [12,16]. In agreement, the genome of *A. ambivalens* contains an open reading frame encoding for a putative subunit B lacking the extra cysteine residue in the 3Fe-binding motif [17]. As previously noticed, these novel enzymes are neither restricted to archaea, nor common to all archaea – for example, *Thermoplasma acidophilum*,

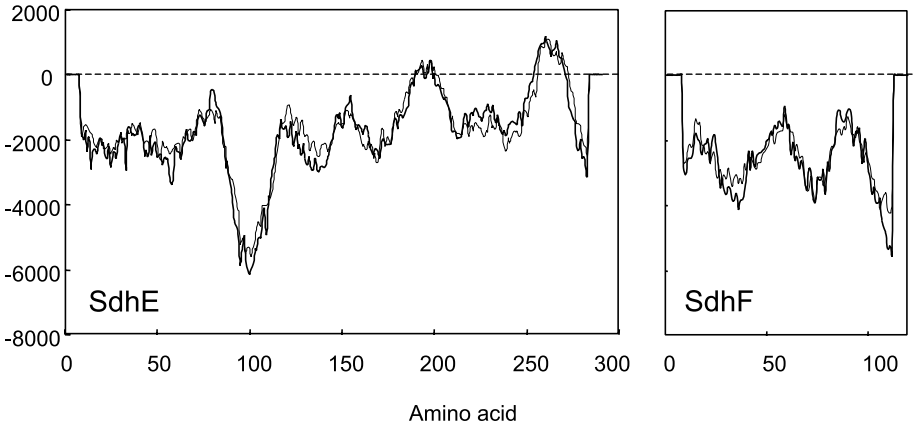


Fig. 3. Hydropathy profiles of SdhE and SdhF from *A. ambivalens*. Hydropathies were determined using TM pred. [48].

Halobacterium halobium, *Archaeoglobus fulgidus* and *Natrobacterium pharaonis* have canonical enzymes, as well as *Sulfolobus metallicus* [18].

The gene clusters for the enzymes of this group do not contain any genes encoding for transmembrane subunits, equivalent to subunit C or D of the first group. Instead, for the archaeal enzymes and for *C. jejuni*, the associated operons contain two other open reading frames coding for two polypeptides of 35 and 14 kDa, that have mainly a hydrophilic character (Fig. 3). In agreement with this genomic organisation, the enzymes from the Sulfolobales, the only ones of this type so far purified, are isolated with four subunits [13,14,19]. Although these subunits were previously also named subunits C and D, it is more convenient to name them subunits E and F, as they are not related at all with the canonical subunits C and D. No homologues for subunit F could be found in the databases, apart from those from *A. ambivalens*, *S. acidocaldarius* and *Sulfolobus solfataricus*. In contrast, homologues of subunit E are widespread, including domains of other enzymes: they are also found in subunit B of heterodisulphide reductase (Hdr) from *Methanobacterium* (*Me.*) *thermoautotrophicum* [20,21], *Aquifex* (*Aq.*) *aeolicus*, *Synechocystis* sp. and *C. jejuni* and as C-terminal extensions of the iron-sulphur subunits from the thiol:fumarate oxidoreductases (Tfr) of *Me. thermoautotrophicum* and *Methanococcus jannaschii*, from the heterodisulphide reductase of *Methanosarcina barkeri* [22] and *Ar. fulgidus*, in glycolate oxidase (Glc) from *E. coli* [23], *B. subtilis, Deinococcus radiodurans* and *Synechocystis* sp. and in anaerobic *sn*-glycerol-3-phosphate dehydrogenase (Glp) from *Escherichia coli* [24], *Aq. aeolicus*, *H. pylori* and *Haemophilus influenzae*.

For the enzymes from *Aq. aeolicus* and *Synechocystis* sp. the genes encoding for the membrane anchors are not found adjacent to those encoding for subunit A or B. However, in the *Synechocystis* genome, there is a gene encoding for a putative protein similar to the archaeal subunit E, which may provide the necessary membrane anchoring (indeed, in *Synechocystis* sp. it has been shown recently that succinate:quinone oxidoreductase activity is clearly membrane associated [25]). However, since the amino acid conservation among the canonical α-helical subunits C and D is very low, it cannot be excluded at present that such subunits are present in those organisms. This question can be solved only upon isolation of these enzymes.

With the exception of sdhE, which possesses only one putative transmembrane helix, close to the C-terminus (residues 256–273 for *A. ambivalens* sdhE) that could provide the necessary membrane anchoring (Fig. 3), all these subunits or domains lack transmembrane helices. At least for *A. ambivalens* SQR [14], *E. coli* anaerobic *sn*-glycerol-3-phosphate dehydrogenase [26] and glycolate oxidase [27], and *Me. thermoautotrophicum* heterodisulphide reductase [21], the enzymatic activities are clearly associated with the membrane fractions. These domains have two other striking characteristics: (i) they contain a duplicated motif rich in cysteines,

$CX_{31-35}CCGX_{38-39}CX_2C$, and (ii) several of the putative helices have a predicted amphipathic nature (Fig. 4).

3.5.1. The cysteine motif in SdhE

This cysteine rich motif is present in a large array of membrane-bound enzymes, performing the most distinct functions, but having always in common its redox activity with quinones (with the exception of thiol:fumarate oxidoreductase). In particular, for the *E. coli* anaerobic *sn*-glycerol-3-phosphate dehydrogenase, it has been shown that the cysteine motif containing subunit is essential for membrane anchoring and for interaction with menaquinone [26].

Interestingly, secondary structure prediction analyses show that the cysteines appear to be located in loops connecting the predicted helices (Fig. 4A), but their function remains unclear. Several hypotheses

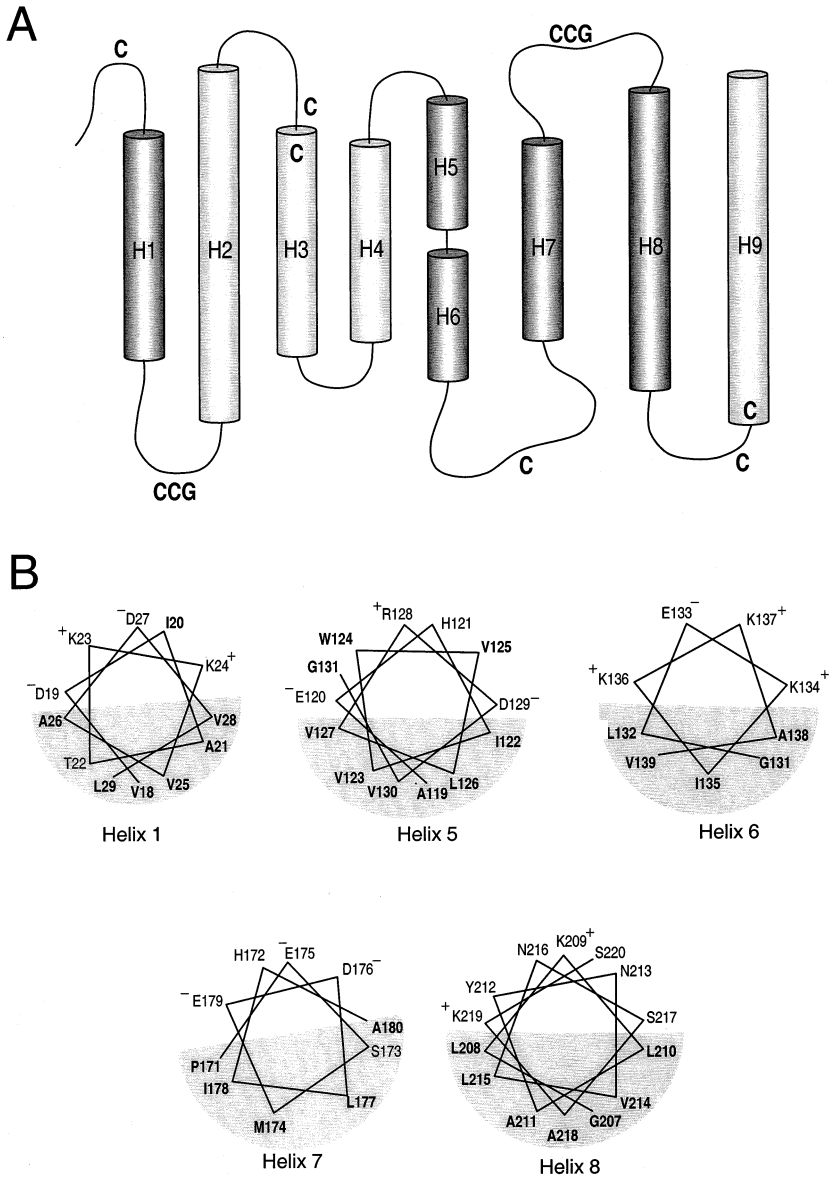
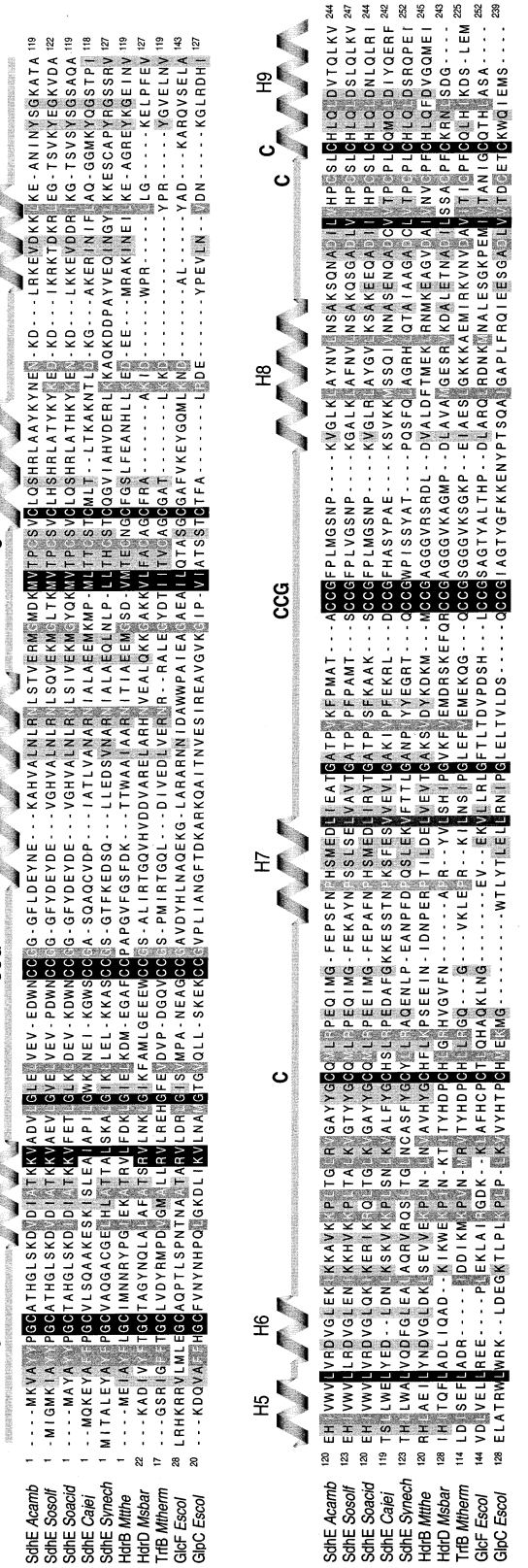


Fig. 4. (A) Representation of the conserved helices in SdhE and homologous peptides (refer to Fig. 1). The amphipathic helices are darker shaded and the relative position of the cysteines of the cysteine motif is represented. (B) Wheel projections of helices 1, 5, 6, 7 and 8 from SdhE of *A. ambivalens*. Amino acid residues containing hydrophobic side chains are depicted in bold and the hydrophobic part of the helices is shaded.



have been proposed: in the case of the heterodisulphide reductase and thiol:fumarate oxidoreductase they could participate in the binding of coenzyme M and coenzyme B [7,28]. For the succinate:quinone oxidoreductases, it was first proposed that they could bind extra iron atoms, but as we demonstrated [14] no additional iron-sulphur centres besides the canonical ones could be detected. Also, iron chemical analysis of the purified *A. ambivalens* enzyme accounted only for the sum of the three iron-sulphur centres. We raised the hypothesis that the cysteines could bind fatty acyl groups, thus providing an alternative process of association to the membrane [14]. However, preliminary tests with the *A. ambivalens* and *S. tokodaii* SQRs suggest that the cysteines are free in the isolated enzymes: a first incubation of the protein with iodoacetamide (IAA) in the presence of SDS and dithiothreitol (DTT) showed that the SdhE subunit, containing the 10 cysteine residues, reacted with IAA, affecting protein mobility [14,20]. If fatty acids were, in fact, bound to the cysteines, a hydroxylamine treatment would generate extra alkylation sites; thus, prior to the DTT/IAA incubation, the protein was incubated with hydroxylamine. The treatment had no effect on SdhE mobility (see Fig. 1 in [13]), indicating that indeed the protein does not bind fatty acids.

In some enzyme complexes, a single repeat of this cysteine motif is also found. Such an example is a putative protein (hmc6) from the large membrane-bound electron transfer complex of the sulphate reducing bacterium *Desulfovibrio vulgaris*, the Hmc (high molecular weight cytochrome *c*, or 16-haem cytochrome) complex [29,30].

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Fig. 5. Multiple sequence alignment of SdhE subunits with homologous peptides. Conserved helices and cysteine motif in all sequences are presented. SdhE *Acamb*, *A. ambivalens*; *Sosolf*, *S. solfataricus*; *Soacid*, *S. acidocaldarius*; *Cajej*, *C. jejuni*; *Synech*, *Synechocystis* sp.; HdrB *Mtthe*, subunit B of *M. thermoautotrophicum* heterodisulphide reductase (7451870); HdrD *Msbar*, subunit D of *Ms. barkeri* heterodisulphide reductase (1890198); TfrB *Mtherm*, subunit B of *M. thermoautotrophicum* thiol:fumarate oxidoreductase (2894538); GlcF *Escol*, subunit F of *E. coli* glycolate oxidase (1707919); GlpC *Escol*, subunit C of *E. coli* anaerobic *sn*-glycerol-3-phosphate dehydrogenase (2506396). Proteins are identified by the gi number assigned by NCBI.

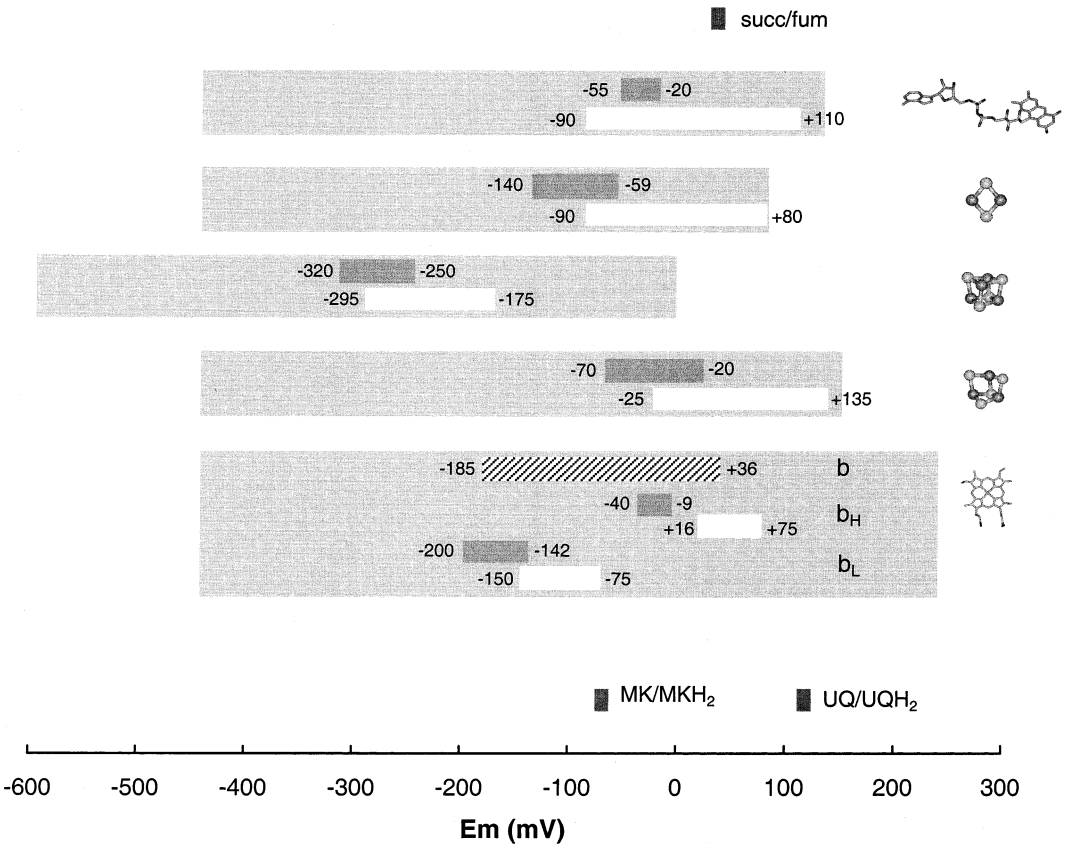

Fig. 6. Reduction potentials of the redox centres in SQRs and QFRs. From top to bottom: succinate/fumarate pair, FAD, centres $[2Fe\text{-}2S]^{2+/1+}$, $[4Fe\text{-}4S]^{2+/1+}$, $[3Fe\text{-}4S]^{1+/0}$, haem *b* (in SQRs where only one haem is present), haems b_L and b_H, and the quinone/quinol pairs relative to menaquinone (MK/MKH_2) and ubiquinone (UQ/UQH_2). The light grey boxes are covering the range of potentials found for the respective type of centre (for the haems, the range shown refers to bis-histidine coordination). The small boxes cover the range of potentials found for the respective centre in QFRs (dark grey) and SQRs (white). The dashed box refers to single haem SQRs. The lowest and the highest values reported are indicated near each box.

3.5.2. The amphipathic helices in SdhE

Membrane proteins use a variety of mechanisms to associate with lipid bilayers. Hydrophobic α-helices and antiparallel β-barrels spanning both leaflets of the membrane are the most known common strategies; these proteins are classified as bitopic, if they span the membrane once, or polytopic, if they span the membrane more than once. A not so well known type of membrane protein are the monotopic ones, which interact only with one side of the membrane [31]. SdhE contains nine putative helices plus a putative transmembrane helix in the C-terminus, and two copies of the $CX_{31-35}CCGX_{39-38}CX_2C$ cysteine motif (Fig. 5). This cysteine motif and helices 1, 2, 3, 5, 7, 8 and 9 are present in all SQR within the same family and is also found in several of the above described proteins. Wheel projections of helices 1, 5, 6, 7 and 8 from *A. ambivalens* SdhE predicts that hydrophobic and hydrophilic amino acids are located on opposite sides of the helices thus suggesting that the protein could interact with the membrane through the hydrophobic face of these amphipathic α-helices (Fig. 4B). Most interesting is the fact that for HdrB*Mtther*, HdrD*Msbar*, TfrB*Mtther*, GlcF*Ecoli* and GlpC*Ecoli* helices 1, 5, and 8 are also amphipathic, suggesting a similar membrane binding strategy. The same type of structure was demonstrated for other monotopic membrane proteins: prostaglandin endoperoxide H synthases (PGHS) 1 and 2 were shown to interact with the membrane via four amphipathic α-helices positioned parallel to the plane of the membrane [32]; the regulators of G protein signalling, RGS4 and RGS16 [33,34], and CTP:phosphocholine cytidyltransferase (CT) [35]

also bind to the membrane through amphipathic α-helices. In particular helices 1, 5 and 8 from *A. ambivalens* SdhE would be particularly suited for this type of membrane anchoring. Besides the hydrophobic sector that interacts with the hydrophobic core of the membranes, these helices contain positive side chains in the interfacial region defined by the lipid head groups, thus promoting electrostatic interactions with anionic lipids present in *A. ambivalens* membranes, as was demonstrated for PGHS, RGS4 and CT. Another interesting characteristic of these amphipathic helices containing positive side chains is that the correct helical fold is only achieved in the presence of anionic lipids [33]; this might explain why, in vitro, the purified *A. ambivalens* complex II is unable of reducing caldariella quinone: this slight misfold would prevent adequate caldariella quinone binding. Correlation of these data leads to the hypothesis that the SdhE peptide of these enzymes anchors the protein monotopically within one leaflet of the membrane, similarly to what may also happen in the other cysteine motif containing proteins. SdhF, as well as SdhE, is hydrophilic (Fig. 3) and also contains amphipathic α-helices (not shown), suggesting that it may also be involved in membrane attachment by a similar strategy.

While subunit E of this type of SQR has one possible hydrophobic α-helix, most of the other enzymes (if not all) containing a similar subunit or domain do not have such a putative anchoring, albeit they are membrane associated, thus reinforcing the hypothesis that binding to the membrane is indeed through amphipathic helices.

4. Redox properties

The succinate/fumarate redox couple has, at pH 7.0, a redox potential of +30 mV, while the electron donors or acceptors, the quinones/quinols, have potentials that range from +110 mV (e.g. ubiquinone) to −74 mV (e.g. menaquinone). Hence, the reduction potentials of the flavin and of several of the metal centres fall in a rather narrow range (Fig. 6), in between the potentials of the acceptor and donor. There are two important exceptions: the redox potentials of the tetranuclear centre and of the low-potential B-type haem (possibly the distal one [36]), which have values considerably lower than those of the other centres (Fig. 6). The reduction potential of the [4Fe-4S] centre substituting for centre S3 in the type E enzymes is presently unknown, albeit that it is an important information to understand the electron transfer mechanism in this subfamily. Also, although in general the QFRs have reduction potentials slightly lower than those of the SQRs, there is a considerable overlap of the potentials reported for both types of enzymes; this explains why the enzymes are bi-directional.

The reduction potentials of the redox centres are well within the ranges found for each type of centre in general (Fig. 6), i.e. they are fine-tuned by the protein backbone to participate in the necessary electron transfer steps of fumarate reduction or succinate oxidation. The substitution of one cysteine ligand by an aspartate in the [2Fe-2S]$^{2+/1+}$ centre has also a negligible effect. The only reduction potential reported for such a centre refers to the *E. coli* SQR, a type C enzyme, and its value (+10 mV [37]) is well inside the range of reduction potentials reported for this centre in SQRs (Fig. 6), being similar to the mitochondrial enzymes from *Bos taurus* (0 mV, [38]) and *Arum maculatum* (−7 mV [39]). Additionally, experiments performed with a mutant of *E. coli* QFR [40] in which the third cysteine ligand of the di-iron cluster was replaced by an aspartate, simulating what occurs in *E. coli* SQR, have shown that the reduction potential of that centre was unchanged in relation to wild-type *E. coli* QFR, −79 mV, a much lower value than that found in *E. coli* SQR.

4.1. Interactions between the centres

The redox centres are linearly arranged, in the order Fv-2Fe-4Fe-3Fe-(b_P-b_D), where the distances between them are approx. 10–14 Å. At such short distances electrostatic interactions are to be expected between all centres (anticooperative in simple electrostatic terms), which will further modulate their actual redox behaviour [41,42]. In fact, for both *R. marinus* SQR and *D. gigas* QFR [10,11], two enzymes of type B, having a single transmembrane helix with two B-type haems, the experimental data for the redox titration of the haems can be simulated with two independent Nernst curves, but the best fitting is only achieved with different haem contributions (e.g. for

D. gigas QFR, at pH 7.6, the haems b_L and b_H have 55% and 45% contribution, respectively). This deviation from the 50/50 contribution is an indication that redox interactions, not only between the haems, but also between b_P (assuming, that for these enzymes $b_P = b_H$ and $b_D = b_L$, as in *B. subtilis* [36]) and the trinuclear centre 3, should be taken into consideration. However, in order to determine the interaction potentials between these centres it is required to follow the individual microstates of the enzyme along the redox potential range, which is not possible with the available experimental data. Thus, only macroscopic reduction potentials of the two haems could be determined by direct analysis of the redox titration curves, as well as for the other enzyme redox centres. An estimation of the intrinsic and interacting potentials can be achieved by a close examination of the existing experimental data for b_L, b_H and centre 3. For *D. gigas* QFR, at pH 7.6, a much better fitting of the experimental haem titration curve was obtained when an anticooperative redox interaction of 30 mV was introduced between b_H and centre 3. Although it is not possible to obtain a unique solution it is clear that redox interactions between the QFR/SQR centres should be taken into account.

4.2. Redox-Bohr effect in the haems

The role of the two centres with a low reduction potential, $[4Fe\text{-}4S]^{2+/1+}$ centre 2 and low-potential haem, has been questioned in thermodynamic terms, as they do not seem to be involved in the main electron transfer steps. However, structural data [9], together with the evidence for the binding of the quinone close to the low-potential haem [36,43], show that they are essential parts of the electron transfer route. Indeed, the apparent uphill electron transfer through these centres may be a key feature to assure directionality of electron transfer: for example, in QFR, as soon as the quinol donates an electron to the low-potential haem, this electron is immediately transferred to the high-potential haem. Thus the first haem becomes available to receive the second electron from the semiquinone. The high potential haem, in its turn, is immediately oxidised by the trinuclear centre, the back transfer rate being quite unfavourable due to the low redox potential of the distal haem; the electron is then transferred to the binuclear centre, again through another centre with a lower potential (centre S2), until it finally reaches the flavin catalytic site. The same reasoning can be applied to the electron transfer in SQR. Through these 'up and down' electron transfer processes, the two electron donating/accepting couples (succinate/fumarate and quinol/quinone) are tightly coupled to the one-electron steps, making the back transfer steps unfavourable. The overall process will be controlled by the succinate/fumarate ratio and by the redox status of the quinone pool. The same reasoning applies for the simpler enzymes, e.g. those having a single haem or no haem at all.

We have recently shown that both haems from the *D. gigas* QFR and, at least, the low-potential haem from *R. marinus* SQR, show a clear redox-Bohr effect, i.e. the reduction potential of the haem(s) depend(s) on the pH, with pK_a values for the oxidised and reduced forms well within the physiological range: $pK_a^{ox} \leq 6.00$ and $pK_a^{red} = 7.7$ for b_L and

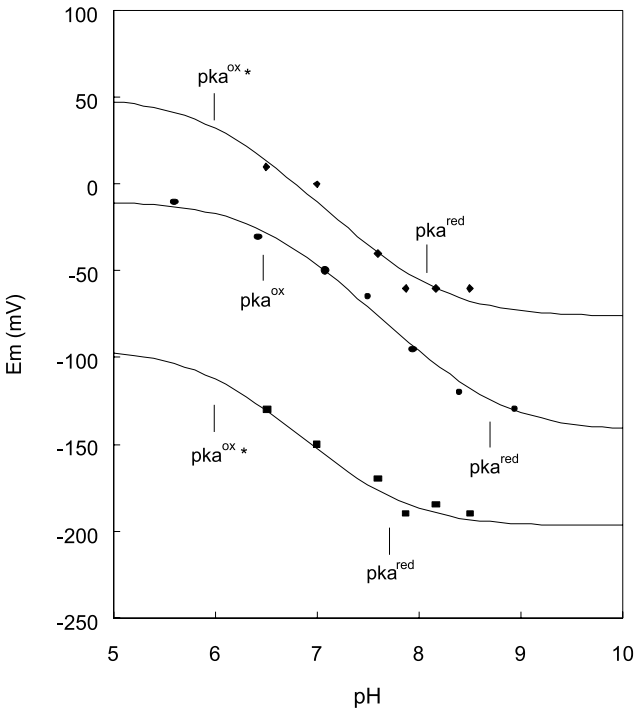

Fig. 7. Redox-Bohr effect on the haem(s) of a QFR and a SQR containing two haems: *D. gigas* QFR haem b_L (■), *D. gigas* QFR haem b_H (♦) and *R. marinus* SQR haem b_L (•). The solid lines were calculated for a single ionization, with the following pK_a^{ox} and pK_a^{red}: 6.0 and 8.1 (■), 6.0 and 7.7 (♦), 6.5 and 8.7 (•). *The experimental values can be equally well fitted with a smaller pK_a^{ox}.

$pK_a^{ox} \leq 6.00$ and $pK_a^{red} = 8.1$ for b_H of *D. gigas* [11] and $pK_a^{ox} = 6.5$ and $pK_a^{red} = 8.7$ for *R. marinus* b_L [10] (Fig. 7). This behaviour is usually due to a direct protonation event at the haem propionates or at a nearby proteolytic residue when the haem iron is reduced. An enzyme with a similar behaviour is the nitrate reductase from *E. coli*, which has a membrane anchor subunit similar to type B SQR and QFR. Recently it was shown that its haem b_L reduction potential also shows a clear dependence on pH of −30 mV/pH unit between pH 5.5 and 8.5 [44].

The pH dependence of the haem reduction potential can have a large implication in the mechanism of action of the different enzymes, namely concerning a possible coupling of electron transfer to proton translocation. Whether the protons involved in the reaction with the quinones are taken up /released to the positive or negative side of the membrane is still uncertain since the number and the location of the quinone/quinol binding sites are still under debate, having large repercussions in the enzyme mechanism and in the energetic balance. The structure from *E. coli* QFR suggests the presence of two quinone binding sites facing opposite sides of the membranes [2], while the QFR from *W. succinogenes* contains a quinone binding pocket, involving a glutamate residue (Glu66), in the periplasmic side close to haem b_D where menaquinone would interact, thus releasing its protons in the periplasm [9]. On the other hand, there is experimental evidence for the *B. subtilis* SQR that haem b_L is located towards the periplasmic side of the cell, thus corresponding to b_D [36] and that succinate oxidation by menaquinone is driven by the electrochemical proton potential [45]. Recently it was observed that fumarate reduction by *B. subtilis* SQR contributes to the formation of a proton gradient [46].

There seems to be consensus now that high reduction potential quinones (e.g. ubiquinone) have their binding sites facing the negative side, picking up protons from the cytoplasm upon reduction, whereas quinones having low reduction potentials (e.g. menaquinone) take protons from the periplasm. Thus, in the first case no alteration in the proton gradient occurs but a change of four H^+ is observed in the gradient in the second case, which can be a gain or a loss depending on whether the quinol is oxidised or the quinone is reduced, respectively. In this case, a redox-Bohr effect as observed for the enzymes from *D. gigas* and *R. marinus* would be of great importance allowing haem b_L (possibly b_D), or a nearby residue, to act simultaneously as electron and proton acceptor/donor of the quinol/quinone, thus being the coupling system.

Overall, it is clear that SQR/QFR are extremely versatile and diverse enzymes, and that even with two crystal structures available many interesting problems remain to be tackled.

Acknowledgements

This work was supported by FCT-Portugal grants 36560/99 and 36558/99 to M.T. R.S.L., A.S.F. and M.M.P. are recipients of PRAXIS XXI grants BD/19867/99, BD/1163/00 and BPD/22054/99, respectively. We would like to thank Prof. António V. Xavier for a most valuable discussion on the redox properties of these enzymes.

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